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Article

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Molecular phylogeny reveals a new species of the *Hygrobates longipalpis* complex from Portugal (Acariformes, Hydrachnidia, Hygrobatidae)

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Abstract
Water mites of the *Hygrobates longipalpis* complex are a common group inhabiting both running and standing waters throughout the Palaearctic. This study provides the first time-calibrated phylogeny of this complex. Using fossil-calibrated molecular dating, we infer that the most recent common ancestor of the *Hygrobates longipalpis* complex originated in the Eocene. The *Hygrobates daochengensis* clade from China represents the earliest-branching lineage, indicating a non-European origin of the Mediterranean members of the *H. longipalpis* complex. *Hygrobates prosiliens*, which inhabits lacustrine environments, diverged from the common ancestral lineage with the remaining European species approximately 21 million years ago. In contrast, the clade comprising *H. longipalpis* and *H. maremagnum* n. sp. appears to be younger, with its diversification possibly triggered by Late Miocene–Pliocene geological events, including the Messinian Salinity Crisis (~10 Ma). The latter species is described here from Portugal as new to science using an integrative taxonomic approach.

Key words Acari, DNA-barcoding, molecular phylogeny, new species.

Introduction

Water mites of the genus *Hygrobates* Koch, 1837 occur in all biogeographic regions except Antarctica (Pešić *et al*. 2017; Smit 2020). Over the last decade, molecular approaches based on DNA barcoding of the mitochondrial cytochrome c oxidase subunit I (COI) gene have challenged traditional taxonomic concepts of most European members of this genus, revealing extensive undescribed cryptic and/or pseudocryptic diversity within morphologically defined species (e.g., *H. fluviatilis* (Ström, 1768): Pešić *et al*. 2017, 2019b, 2020a, 2023a; *H. calliger* Piersig, 1896: Pešić *et al*. 2021, 2022b; *H. longiporus* Thor, 1898: Pešić *et al*. 2021, 2022a, 2024; *H. nigromaculatus* Lebert, 1879: Martin *et al*. 2010; Pešić *et al*. 2020b; *H. trigonicus* Koenike, 1895: Pešić *et al*. 2021; Pešić & Esen 2022).

Hygrobates longipalpis (Hermann, 1804) was long regarded as a common inhabitant of standing and slow-flowing waters and was listed in the water mite literature (e.g., Gerecke *et al*. 2016) as having a Holarctic distribution. However, a more recent molecular study by Pešić *et al*. (2019a) demonstrated that European populations attributed to *H. longipalpis* comprise two distinct lineages: *H. prosiliens* Koenike, 1915, occurring in stagnant waters, and *H. longipalpis* sensu stricto, preferring slow-flowing sectors of running waters. The taxonomic status of non-European populations previously published under *H. longipalpis* sensu lato remains unclear and requires reassessment using molecular techniques (Pešić *et al*. 2023b). Recently, Li *et al*. (2025) described a new species from China belonging to the *H. longipalpis* complex, *H. daochengensis* Li & Pešić, 2025.

As part of recent DNA barcoding initiatives conducted within the Biodiversity Genomics Europe (BGE; https://biodiversitygenomics.eu/) project, a number of COI sequences from specimens collected in Portugal and morphologically assigned to the *H. longipalpis* species complex have been generated. This dataset provides an opportunity to reconstruct the diversification history of the complex using a time-calibrated phylogeny and to clarify the taxonomic status of Portuguese populations. As a result of this study, one new species of *Hygrobates* is described herein.

Material and Methods

Water mites were collected by hand netting, sorted live in the field, and immediately preserved in 96% ethanol. Specimens for molecular analysis were examined and photographed without dissecting under a compound microscope in ethanol. Images and field metadata were uploaded in BOLD SYSTEMS public database. After DNA extraction, some specimens were dissected and slide mounted in Faure's medium.

Morphological nomenclature follows Gerecke *et al*. (2016); for explanations concerning morphology and measurements of *H. fluviatilis*-complex see Figs. 1B-D in Pešić *et al*. (2017). The genital acetabula in both sexes and the genital plate in the female were measured on both sides, and therefore their dimensions are given as a range. The holotype and paratypes of the new species will be deposited in the Naturalis Biodiversity Center in Leiden (RMNH).

All measurements are in µm. The following abbreviations are used: Ac-1 = most anterior acetabulum; Cx-I = first coxae; dL = dorsal length; H = height; I-L-4-6 = fourth to sixth segments of first leg; L = length; Ma (mega-anum) = million years; mL = median length; MRCA = the most recent common ancestor; n = number of specimens examined; P-1–P-5 = palp segments 1 to 5; W = width.

Molecular analysis

The molecular analysis was conducted at the University of Florence (Florence, Italy). DNA was extracted using a non-destructive protocol. For the methods used for cytochrome c oxidase subunit I (COI) gene amplification and sequencing, see Pešić *et al*. (2024). Raw reads were demultiplexed using the Pacific Biosciences SMRT Link software. Consensus sequences were generated with the PacBio Amplicon Analysis (pbaa) tool. Primer trimming, translation and stop codon checking were performed using Geneious Prime 2024.0.1. Consensus sequences were made available in the Barcode of Life Data Systems (BOLD). Relevant voucher information, photos, and recently generated DNA barcodes are publicly accessible through the Dataset - DS-BGEPL05 (BGE Biodiversity Genomics Europe:

Portuguese water mites V - Molecular phylogeny of *Hygrobates longipalpis* complex) in BOLD (dx.doi.org/10.5883/DS-BGEPL05).

In this study DNA was extracted from ten specimens of the *Hygrobates longipalpis* complex from Portugal listed in Table 1. For all other species, COI sequence data were taken from Pešić *et al*. (2019a, 2023b) and Li *et al*. (2025) and downloaded from the respective sequence data archives. In total we used a COI dataset with 105 sequences of *Hygrobates longipalpis* complex, representing COI haplotypes of *H. longipalpis* (n=75), *H. prosiliens* (n=19) and *H. daochengensis* from China (n=1) for phylogenetic analyses.

Table 1. Details on DNA barcoded specimens of *Hygrobates maremagnum* **n. sp**. from Portugal, including coordinates of sampling sites, the barcode index number and associated data obtained from BOLD. BOLD data presented here was last accessed on 15th December 2025.

Locality	Coordinates	Sample ID	Process ID	BIN
Setúbal, Montijo, Santo Isidro de Pegões, Montante	38.644° N, 8.615° W	CCDB_39397_B03	HYDAS015-22	BOLD:AEU0047
Guarda, Seia, Praia Fluvial de Sabugueiro	40.401° N, 7.640° W	BGE_00227_B04	BSNTN966-23	
Guarda, Seia, Nossa Senhora do Desterro	40.395° N, 7.694° W	BGE_00108_F07	BBIOP162-24	
Guarda, Seia, CISE, fountain	40.419° N, 7.709° W	BGE_00227_B09	BSNTN971-23	
Guarda, Seia, Covão do Forno	40.369° N, 7.638 ° W	BGE_00227_D10 BGE_00108_A04	BSNTN996-23 BBIOP099-24	
Porto, Lousada, Moinho da Tapada	41.263° N, 8.307° W	BGE_00109_E04	BBIOP052-24	
Guarda, Gouveia, Barragem do Vale do Rossim	40.400° N, 7.589° W	BGE_00108_C06	BBIOP125-24	
Guarda, Seia, Covão do Forno	40.369° N, 7.638° W	BGE_00108_A05	BBIOP100-24	BOLD:AFT9706
Guarda, Gouveia, Barragem do Lagoacho	40.385° N, 7.618° W	BGE_00108_C09	BBIOP128-24	

Sequence comparisons were performed using MUSCLE alignment (Edgar 2004). Intra and interspecific genetic distances were calculated based on the Kimura 2-parameter (K2P) model (Kimura 1980), using MEGA 11 software (Tamura *et al*. 2021). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Additionally, the sequence data were analysed using the Assemble Species by Automatic Partitioning (ASAP) method (Puillandre *et al*. 2021). We used the online ASAP version available at iTaxotools (https://itaxotools.org/index.html) with default settings and K2P distance model.

Phylogenetic analysis

The TCS haplotype network was constructed for resolving relationships between species of the *Hygrobates longipalpis* complex based on the trimmed COI dataset of 56 haplotypes (n=108) with the final length 566 bp. using POPArt (http://popart.otago.ac.nz) (Clement *et al*. 2002; Leigh & Bryant, 2015).

The 3-partitioned phylogeny was based on the COI dataset with 72 sequences of *Hygrobates* spp. Representatives of Hydrachnoidea (*Hydrachna conjecta* and *H. globosa*) were used as outgroup. Maximum Likelihood phylogenetic analysis was performed using IQ-TREE version 3 (Wong *et al*. 2025) with automatic identification of the most appropriate evolutionary models (Chernomor *et al*. 2016) and ultrafast bootstrapping algorithm (UFBoot) with 5000 replicates (Hoang *et al*. 2018). Models of sequence evolution for each partition calculated through Model Finder (Kalyaanamoorthy *et al*. 2017) based on Bayesian Information Criterion (BIC) were as follows: 1st codon of COI: TN + G; 2nd codon of COI: TN + I; and 3rd codon of COI: HKY + G.

Divergence time estimates

For the time-calibrated COI phylogeny, we used a dataset with 5 *Hygrobates* species. Additionally, representatives of Hydrachnoidea (*Hydrachna conjecta* and *H. globosa*) and Arrenuroidea (*Horreolanus orphanus* and *Mideopsis roztoczensis*) were included to add a calibration point and outgroup. Models of sequence evolution for each partition of this dataset were also calculated through Model Finder (Kalyaanamoorthy *et al*. 2017) based on Bayesian Information Criterion (BIC) and were as follows: 1st codon of COI: HKY + G; 2nd codon of COI: TIM3 + I; and 3rd codon of COI: F81 + I. Instead of using the estimated best-fit models, we used the less complex HKY model with corresponding distributions for each partition.

Calculations were performed in BEAST v2.7.4 with an optimized relaxed clock and Yule speciation process as priors (Bouckaert *et al*. 2019). We used external mean rate 0.0177±0.0019 of COI partition dataset calculated for insects by Papadopoulou *et al*. (2010). We also included one fossil calibration point for MRCA of Arrenuroidea, Miocene (Cook 1957; Dabert *et al.* 2016) with an exponential distribution (mean (lambda) = 3.11, offset = 11.5).

Two independent runs of 25,000,000 generations were implemented, with sampling every 1000 generations. The resulting tree sets were combined using LogCombiner v2.7.4 with 10% burn-in. The final ESS values were checked using Tracer v1.7 (Rambaut *et al*. 2018) and each value was recorded as >400. A maximum clade credibility tree has been computed with TreeAnnotator v2.7.4.

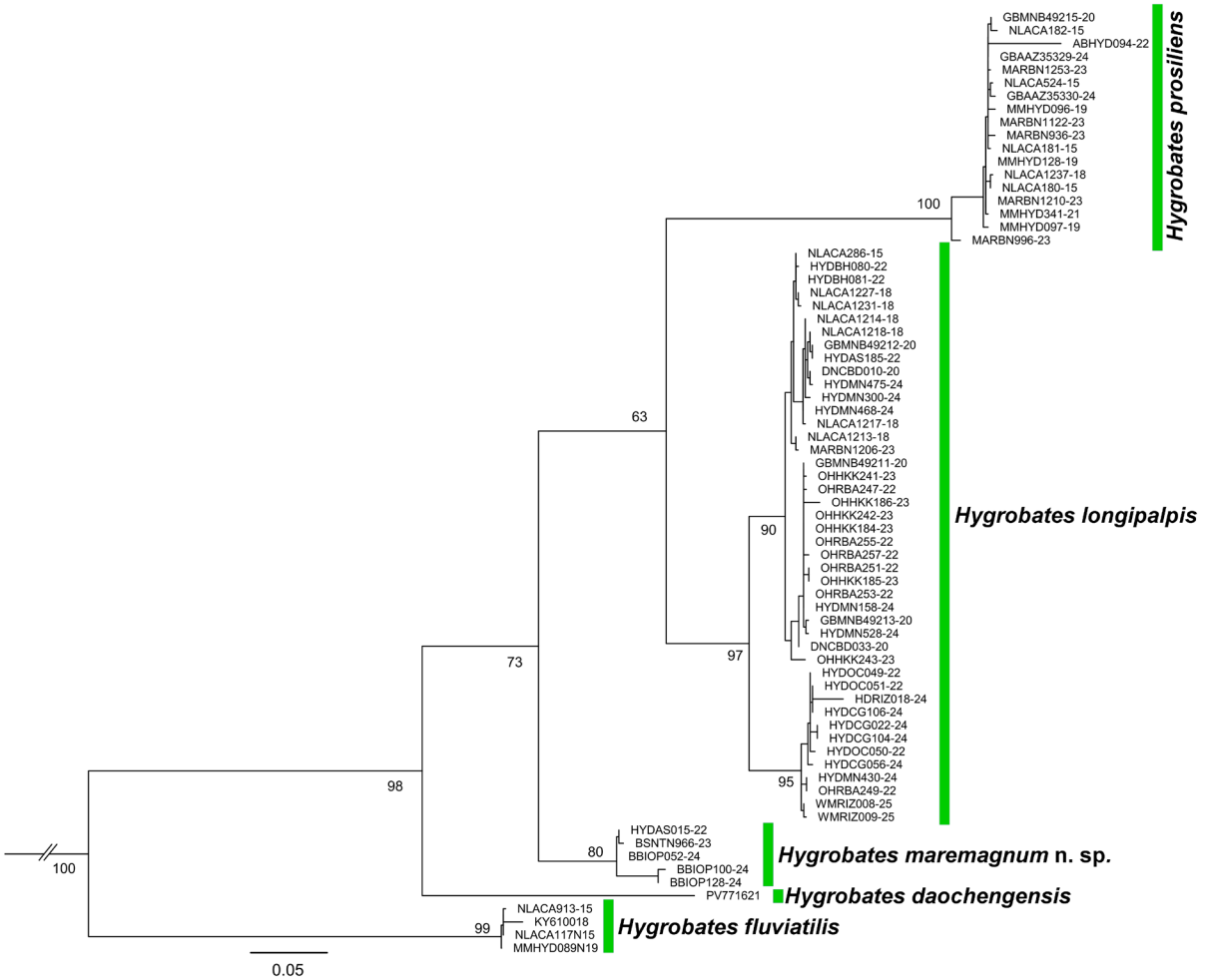

Figure 1. Maximum Likelihood (ML) phylogeny of the COI sequence data set of the *Hygrobates longipalpis* complex. Scale bar indicates the branch lengths. Black numbers near nodes are ML ultrafast bootstrap support values (BS). Outgroup is not shown.

Results

Phylogenetic analysis

The COI phylogenetic analyses and TSC network (Figures 1-2) revealed four species-level clades in the *Hygrobates longipalpis* complex, i.e. three previously known and described species, *H. longipalpis* and *H. prosiliens* from the Western Palaearctic and *H. daochengensis* from China, and one undescribed lineage from Portugal, here described as *H. maremagnum* **n. sp**. Studied lineages were highly to moderately supported (BS = 80–100) in the ML analysis. *Hygrobates maremagnum* **n. sp**. was revealed as a sister clade to *H. longipalpis* and *H. prosiliens* but with moderate supports. *Hygrobates longipalpis* in both ML and network analyses is divided into two subclades/lineages (Figure 2), with lineage 1 being represented by specimens from several countries from Norway to the Balkans and lineage 2 being represented by mostly Turkish specimens.

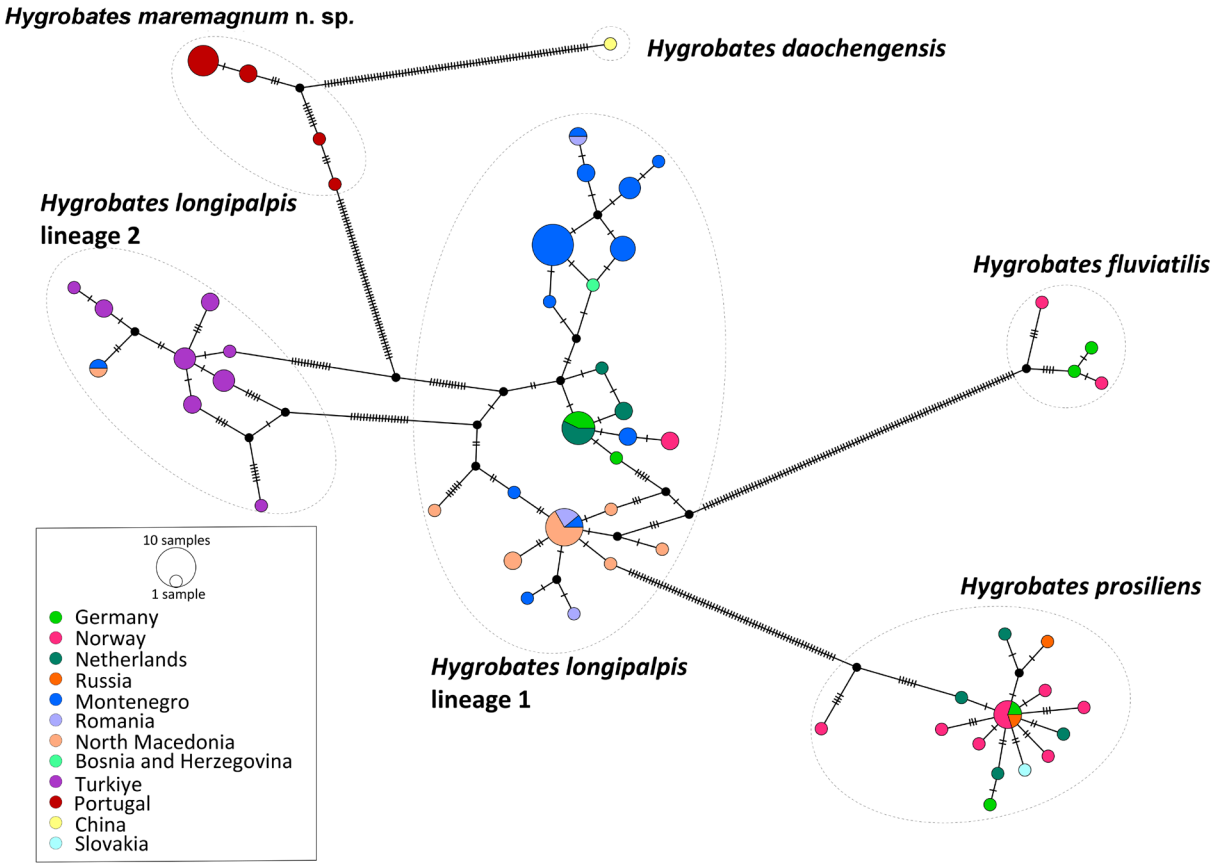

Figure 2. TCS network based on the COI dataset of *Hygrobates longipalpis* complex (n = 108). Hatch marks on the branches indicate the number of nucleotide substitutions between haplotypes. Size of circles corresponds to the number of available sequences for each haplotype (smallest circle = one sequence).

Table 2. Estimates of average genetic distance (K2P) within and between COI clades examined of the *Hygrobates longipalpis* complex.

Clades	Intraspecific distances	Interspecific distances		
		(1)	(2)	(3)
(1) *Hygrobates longipalpis*	0.0317			
(2) *Hygrobates prosiliens*	0.0152	0.170		
(3) *Hygrobates maremagnum* **n. sp**.	0.0114	0.147	0.188	
(3) *Hygrobates daochengensis*	n/c	0.207	0.216	0.185

The average genetic distance between COI sequence groups recovered in the K2P analysis ranged from 14.7% between *Hygrobates longipalpis* and *H. maremagnum* **n. sp**., to 21.6% between *H. prosiliens* and *H. daochengensis* (Table 2). These genetic distances were higher than the barcoding gap found by the ASAP method (7% to 13%; Figures 3A-B), which supports the species-status of the new species. The mean intraspecific distance within clades ranged from 1.14% K2P in *H. maremagnum* **n. sp**. to 3.17% K2P in *H. longipalpis*.

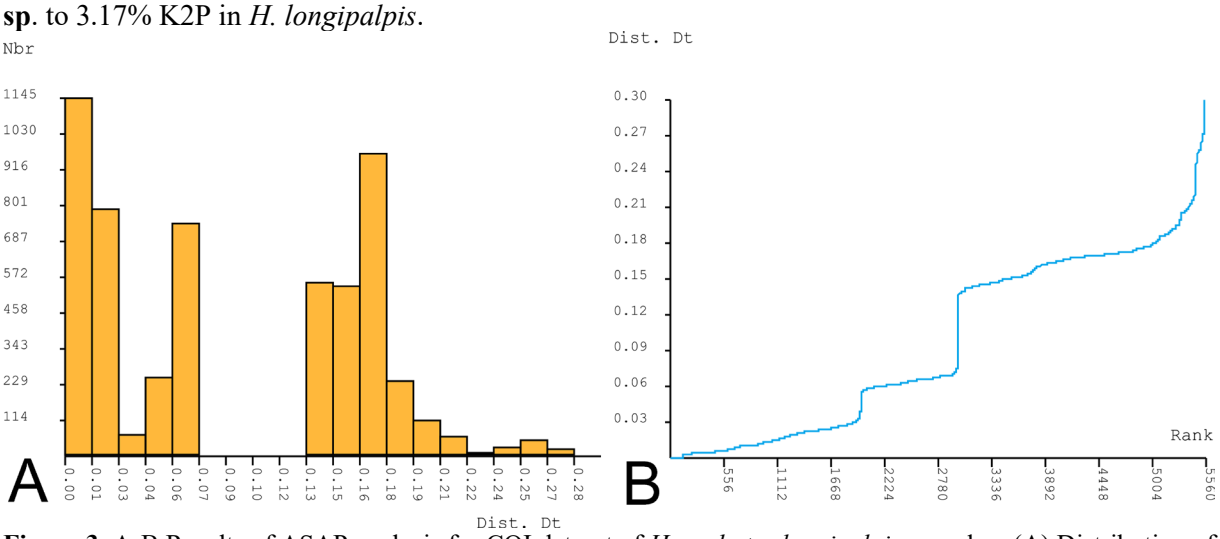

Figure 3. A-B Results of ASAP analysis for COI dataset of *Hygrobates longipalpis* complex. (**A**) Distribution of pairwise differences, (**B**) Ranked pairwise differences.

Time-calibrated reconstruction of phylogeny

Time-calibrated COI phylogeny (Figure 4) revealed that the MRCA of *Hygrobates longipalpis* complex likely originated in the Eocene (mean age = 42.6 Ma, 95% highest posterior densities (HPD) = 23.4-73.4 Ma, BEAST BPP = 0.99). *Hygrobates daochengensis* from China represents the basal clade for this group. The diversification within the *Hygrobates longipalpis* complex possibly began from this time and continued during the Oligocene-Miocene. A divergence of *H. longipalpis* lineages is dated by Pleistocene (mean age = 2.6 Ma, 95% HPD = 1.2-5.2 Ma, BEAST BPP = 1.00).

Systematic descriptions

Family Hygrobatidae Koch, 1842

Genus *Hygrobates* Koch, 1837
Subgenus *Hygrobates s.s.*

***Hygrobates maremagnum* Pešić n. sp.**
https://www.zoobank.org/urn:lsid:zoobank.org:act:EBBD47DA-0A25-4F44-9EE8-5A6694DDC1D6
Figs. 5-6

Material examined — Holotype ♂ (Sample ID: BGE_00227_B04), sequenced (BSNTN966-23), dissected and slide mounted (RMNH), **Portugal**, Guarda, Seia, Praia Fluvial de Sabugueiro, Alva River, 40.401° N, 7.640° W, 1021 m asl., 24 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha. Paratypes: 1♀ (Sample ID: BGE_00108_F07), sequenced (BBIOP162-24), dissected and slide mounted (RMNH), Guarda, Seia, Nossa Senhora do Desterro, Alva river, 40.395° N, 7.694° W, 791 m asl., 23 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha; 1♂ (Sample ID: BGE_00108_A04), sequenced (BBIOP099-24), dissected and slide mounted (RMNH); Guarda, Seia, Covão do Forno, 40.369° N, 7.638° W, 1574 m asl., 21 Aug. 2023, leg. Ferreira, Benitez-Bosco, & Padilha; 1♂ (Sample ID: BGE_00108_C06), sequenced (BBIOP125-24), Guarda, Gouveia, Barragem do Vale do Rossim, Fervença stream, 40.400° N, 7.589° W, 1418 m asl., 22 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha; 1♀ (Sample ID:

BGE_00108_C09), sequenced (BBIOP128-24), Guarda, Gouveia, Barragem do Lagoacho, Covão do Urso stream 40.385° N, 7.618° W, 1438 m asl., 22 Aug. 2023, leg. Ferreira, Benitez-Bosco & Padilha; 1♀ (Sample ID: BGE_00109_E04), sequenced (BBIOP052-24), Porto, Lousada, Moinho da Tapada, 41.263° N, 8.307° W, 176 m asl., 1 Sept. 2023, leg. Ferreira, Sousa, Oliveira & Girão.

Diagnosis — *Morphological*. Posteromedial margin of Cx-I triangular, not tongue-shaped; P-4 distinctly protruding near insertions of the ventral setae. *Molecular*. This lineage is represented by two unique BINs (BOLD:AEU0047, BOLD:AFT9706) that differ from *Hygrobates longipalpis* by 14.7% K2P for COI DNA barcodes.

Description. *General features.* Both sexes — Colour yellowish with pale to dark brown spots. Posteromedial margin of Cx-I triangular (Figure 5A); Cx-IV with a distinct nose-like protruding medial margin, occasionally indented at transition to posterior margin. Acetabula rounded, in triangular arrangement. Basal segment of chelicera dorsally with a pointed projection (Figure 6B). P-2 distoventrally protruding in a short, rounded projection covered by small denticles, P-3 with denticles covering distal two thirds of ventral margin, P-4 distinctly protruding near insertions of the ventral setae (Figures 5B-C), ventral setae in distal parts. *Male* — Anterior margin of genital field convex with a small knob-shaped medial projection, posterior margin indented, in the centre of the indentation with a small protrusion not extending beyond posterior plate margin (Figure 6A); P-4 L/H ratio 4.0–4.2. *Female* — Medial margin of the genital plate strongly indented in the centre (Figure 6C); P-4 slender than in male, L/H ratio 4.3.

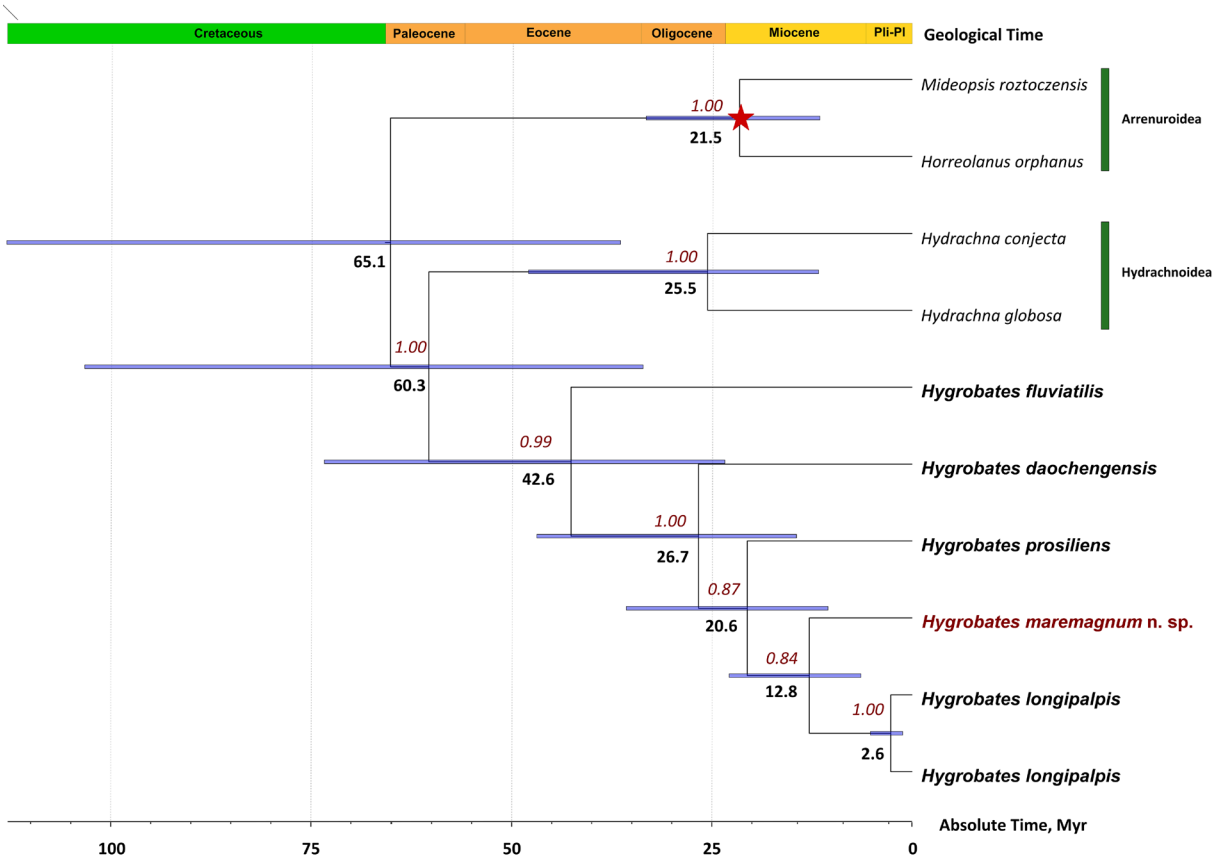

Figure 4. Time-calibrated phylogeny of *Hygrobates longipalpis* complex based on the COI dataset (three codons of COI) reconstructed using external COI rate for insects (Papadopoulou *et al*. 2010) and one fossil record for Arrenuroidea (Cook 1957; Dabert *et al*. 2016). Calibration point is marked by red star. Red numbers above the nodes are Bayesian posterior probability (BPP) values from BEAST v. 2.7.4. Black numbers under the nodes are the mean node ages (Ma). Node bars are 95% HPD of divergence time. Stratigraphic chart according to the International Commission on Stratigraphy, 2024 (https://stratigraphy.org/chart).

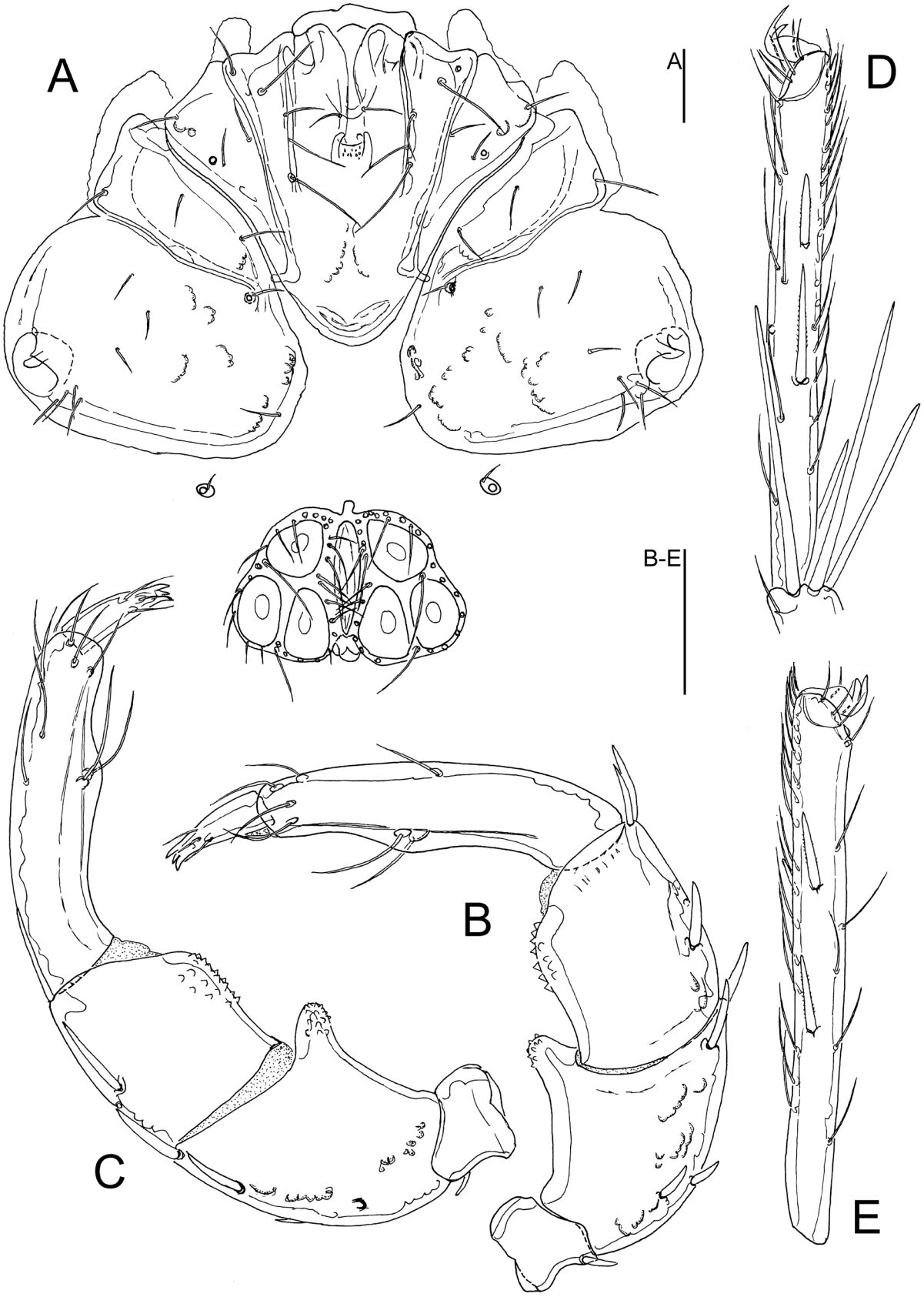

Figure 5. *Hygrobates maremagnum* **n. sp.** ♂ (A-B, D-E holotype; C paratype): A – coxal and genital field; B – palp, lateral view; C – palp, medial view; D-E – IV-L-6. Scale bar = 100 μm.

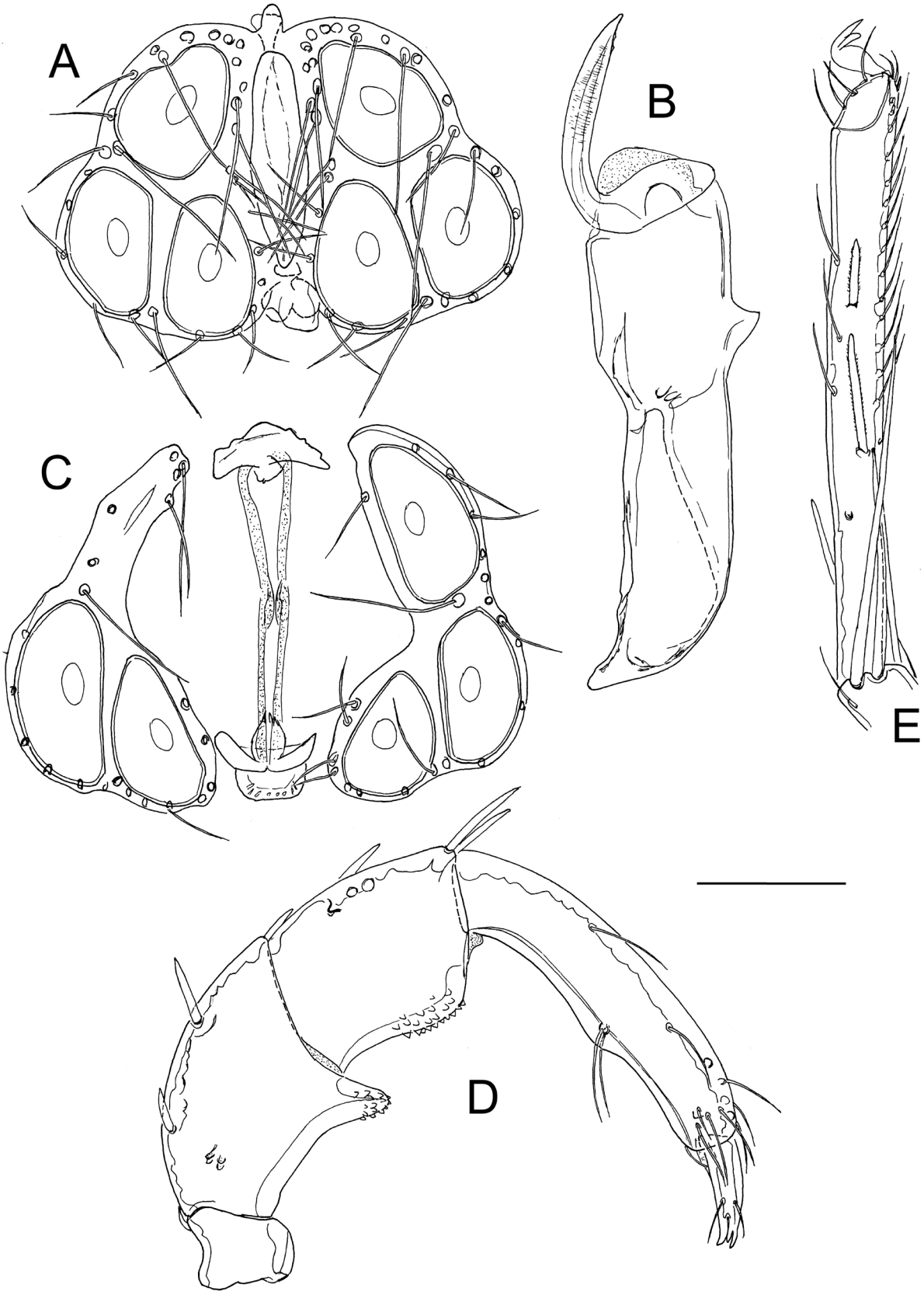

Figure 6. *Hygrobates maremagnum* **n. sp.** (A-B, ♂ holotype; C-D, ♀ paratype): A, C – genital field; B – chelicera; D– palp, medial view; E – IV-L-6. Scale bars = 100 µm.

Measurements — *Male* (holotype; BGE_00227_B04; in parentheses measurements of paratype BGE_00108_A04) — Idiosoma L 1280 (1115), W 1200 (1070); coxal field: L 603 (588); Cx-III W 712 (697); mL of Cx-I + gnathosoma L 450 (436); distance between lateralmost ends of Cx-II apodemes, 219. Genital field L/W 230/320 (225/319), ratio 0.72 (0.71); L gonopore 149 (159); L Ac 1-3: 97 (91-95), 98-102 (100), 97-103 (98-103). Ejaculatory complex L 216 (208).

Palp — Total L 725 (711), dL/H: P-1, 47/59 (46/66); P-2, 200/125 (194/125); P-3, 147/114 (137/109); P-4, 253/63 (253/60); P-5, 78/27 (81/25); dL P-2/P-4 ratio 0.79 (0.77). Chelicera total L 476, basal segment L 316 (306), claw L 156 (147).

Legs — dL of I-L: 100 (100), 150 (147), 241 (241), 319 (319), 337 (328), 264 (274). dL of IV-L: 231 (206), 219 (225), 356 (356), 494 (494), 456 (475), 384 (381); IV-L-6 proximomedial seta L 53-78 (69), distomedial seta L 53-48 (66), proximomedial/distomedial seta L ratio 1.0-1.63 (1.05).

Female (paratype; BGE_00108_F07) — Idiosoma L 1310, W 1240; mL of Cx-I + gnathosoma L 455; distance between lateralmost ends of Cx-II apodemes, 244. Genital field L/W 264/358; genital plate L 259; pregenital sclerite W 80; gonopore L 222; L Ac 1-3: 115, 106, 88. Egg maximum diameter (n=2) 208-215.

Palp — Total L 736; dL/H: P-1, 48/63; P-2, 198/120; P-3, 144/116; P-4, 270/63; P-5, 76/25; dL P-2/P-4 ratio 0.73. Chelicera claw L 163.

Legs — dL of I-L: 106, 150, 253, 331, 344, 291. dL of IV-L: 213, 225, 369, 513, 494, 430. IV-L-6 proximomedial seta L 78, distomedial seta L 44, proximomedial/distomedial seta L ratio 1.77.

Etymology — From *Mare Magnum* (Latin for "Great Sea"), a name used by the Romans to describe the Mediterranean.

Discussion — Phylogenetic analysis based on COI sequence data places *Hygrobates maremagnum* **n. sp**. as the sister species of *H. longipalpis*, which it resembles in the setation of the hind leg (IV-L-6 bearing a long proximomedial seta) and in its preference for slow-flowing sectors of running waters. Morphologically, the new species can be distinguished from *H. longipalpis* by the more projecting, tongue-shaped posteromedial margin of Cx-I+II (shortened in the new species) and by the less developed ventral swelling on P-4. The high genetic distance between *H. longipalpis* and *H. maremagnum* **n. sp**. (COI: 14.7% K2P) suggests a long history of independent evolution of the new species.

BIN assignments — The sequenced specimens from Portugal clustered within two BINs. The first cluster, BOLD:AEU0047, includes, in addition to specimens from Portugal, four private sequences of unidentified *Hygrobates* specimens from Italy and three specimens from Spain assigned to *H. longipalpis*. The *p*-distance between this BIN and its nearest neighbour, BOLD:AFU1558, which includes one private sequence of an unidentified *Hygrobates* specimen from Sicily, was estimated at 1.84%. The second cluster (BOLD:AFT9706) includes two specimens from the present study, shows a *p*-distance of 2.56% to its nearest neighbour, BOLD:AEU0047.

Malformations — In the mounted female paratype, one acetabulum was missing on one side of the genital plate (Figure 6C).

Distribution — Portugal (Guarda and Porto districts), Spain, Italy. The detailed distribution of the new species remains uncertain due to its previous confusion with *H. longipalpis*. However, the current distribution of its BINs suggests that the species is probably widespread in the Western and Central Mediterranean region. Former records of *Hygrobates longipalpis* from Portugal (Lundblad 1956) should likely be reassigned to the new species.

Discussion

For a long time, *Hygrobates longipalpis* was considered as a common Palaearctic species, lacking well-differentiated morphological pathways among populations from geographically isolated areas or different habitats (e.g., standing versus slowly running waters). However, the study by Pešić *et al*. (2019a), supported by the results presented here, demonstrated the presence of distinct genetic lineages exhibiting substantial COI sequence divergence. Over approximately 43 million years, the *H. longipalpis* species complex has diversified into at least three species currently recognized in the western Palaearctic, with a fourth species, *H. daochengensis*, recently described from China (Li *et al*. 2025).

Despite their deep genetic divergence, these species are separated by only relatively minor morphological differences.

Morphological stasis, whereby species or lineages exhibit little observable morphological change over long evolutionary timescales (Korshunova *et al*. 2019), is generally attributed to low genetic variation, developmental or genetic constraints, and/or ecological stability that limits selection on relevant traits (e.g., Struck *et al*. 2018). In the species pair *H. longipalpis–H. prosiliens*, cryptic lineages are clearly segregated by habitat, with *H. prosiliens* occurring in standing waters and *H. longipalpis* preferring slow-flowing sectors of running waters. In contrast, the species pair *H. longipalpis–H. maremagnum* **n. sp**. appears, at first glance, to show overlapping habitat preferences, as both taxa inhabit slow-flowing waters. However, across all studied localities in Portugal, only *H. maremagnum* **n. sp**. was recorded. This pattern may indicate a long-term geographical isolation, leading to the accumulation of standing genetic variation, which includes not only ancestral morphological traits retained prior to divergence, but also variants introduced through historical gene flow among closely related lineages (Schluter & Rieseberg 2022).

Fossil-calibrated modelling in this study indicates that the most recent common ancestor of the *Hygrobates longipalpis* complex originated in the Eocene, with the *H. daochengensis* clade representing the earliest-branching lineage. The divergence between the Chinese and European lineages appears to have occurred in the early Miocene (ca. 27 Ma). This finding supports the hypothesis of an Asiatic, or at least non-European, origin of the members of the *H. longipalpis* complex inhabiting the Mediterranean region. Under this scenario, European representatives of the *H. longipalpis* complex may be regarded as relicts of a westward dispersal of the ancestral lineage through the Pannonian Channel during the early Miocene. This channel has been considered a potential dispersal route for freshwater fauna from Asia and Central Europe to the Mediterranean prior to the fragmentation of the residual Tethys into the Paratethys (Sarmatian) and the Mediterranean Basin during the late Middle Miocene (ca. 12.65 Ma) (Bănărescu 1992; Penzo *et al*. 1998).

Figure 7. Photograph of rio Alva, the type locality (Praia Fluvial de Sabugueiro, Seia, Guarda, Portugal) of *Hygrobates maremagnum* **n. sp**. Photo by S. Ferreira.

Divergence within European clades began in the early Miocene (ca. 21 Ma) and continued throughout the Miocene, leading to the differentiation of species. *Hygrobates prosiliens* diverged from the common ancestral lineage of the other European species around 21 Ma, whereas the clade comprising *H. longipalpis* and *H. maremagnum* **n. sp**. appears to have originated approximately 13 Ma. This suggests that the ancestor of this clade was already present in the Middle Miocene, and that the subsequent diversification into distinct species was possibly triggered by Late Miocene–Pliocene geological events, including the Messinian Salinity Crisis (~10 Ma). Diversification within the *H. maremagnum–H. longipalpis* clade was likely driven by colonization of slow-flowing waters by the (lacustrine) ancestor of the clade, combined with dramatic environmental changes during the Messinian Salinity Crisis, an event often considered a key driver of diversification in Mediterranean freshwater fauna (e.g., Penzo *et al*. 1998; Hupało *et al*. 2020).

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